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(54) Title Synthetic vaccine for the specific induction of cytotoxic T-lymphocytes

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Description

Synthetic vaccine for the specific induction of cytotoxic T-lymphocytes

The present invention relates to a synthetic vaccine for the specific induction of cytotoxic T-lymphocytes.

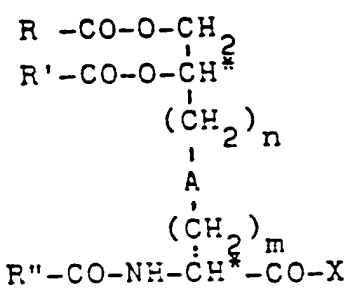
Cytotoxic T-lymphocytes (killer T-cells) are an essential part of the immune response of warm-blooded animals against intracellular infections. Cytotoxic T-lymphocytes are normally induced only by means of an in vivo vaccination with infectious pathogens (J. Bastin et al., J. Exp. Med., Vol 165, June 1987). Because of the risks associated with this, a synthetic vaccine for the specific induction of cytotoxic T-lymphocytes would be a substantial improvement. Surprisingly it has now been found that the specific in vivo induction of cytotoxic T-lymphocytes is possible by the use of certain membrane anchor/active compound conjugates containing killer T-cell epitopes.

Although it has been known that membrane anchor/active compound conjugates are suitable for generating neutralizing antibodies (cf. Angew. Chem. 97 (1985), No. 10, p 883 ff.), a synthetic vaccine containing membrane anchor/active compound conjugates for the specific induction of cytotoxic T-lymphocytes has not yet been reported.

The invention therefore relates to a synthetic vaccine for the induction of cytotoxic T-lymphocytes which comprises a conjugate of at least one membrane anchor compound and a protein, containing at least one killer T-cell epitope, of a virus, a bacterium, a parasite or a tumor antigen, or at least one partial sequence containing at least one killer T-cell epitop of a viral,

bacterial or parasite prot in or of a tumor antigen.

The said membrane anchor compound preferably is a bacterial lipoprotein. A compound of the formulae below is particularly preferred as membrane anchor compound



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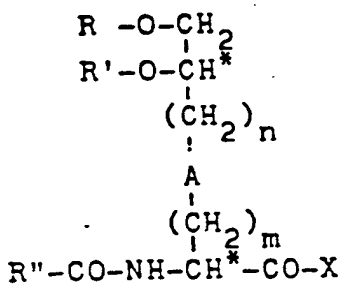

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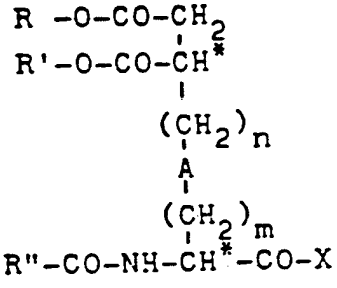

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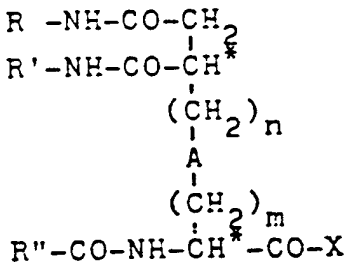

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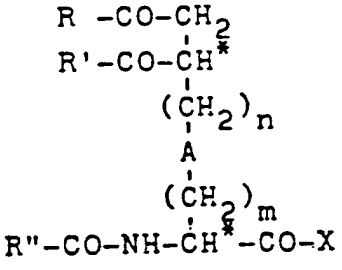

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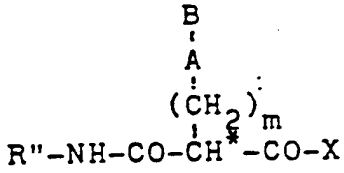

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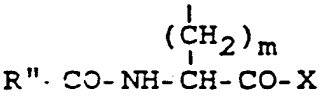

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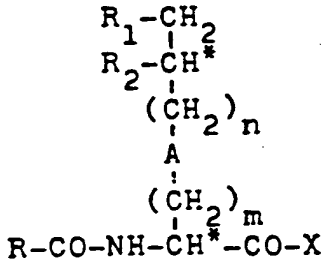


VII.

IX.

in which A may be sulfur, oxygen, disulfide (-S-S-), methylene ($-CH_2-$) or -NH-;

n = 0 to 5, m = 1 or 2;
C* is an asymmetric carbon atom with an R- or S-configuration,
R, R' and R" are identical or different and are hydrogen or an alkyl, alkenyl or alkynyl group having 7 to 25 carbon atoms, which can be substituted with hydroxyl, amino, oxo, acyl, alkyl or cycloalkyl groups, E in formula IX can be hydrogen or any desired side chain of a natural or artificial α-amino acid, B in formula VI can have the meaning of each of the $-(CH_2)_n-$(substituted alkyl) radicals listed in the formulae I - V, and R_1 and R_2 are identical or different and have the same meanings as R, R' and R" but can also be -OR, -O-COR, -COOR, -NHCOR or -CONHR, where X is a chain of up to 10 amino acids to which the protein or the partial sequence of the viral, bacterial or parasite protein or of a tumor antigen is bonded, or is the protein or the partial sequence itself.

Examples of these which may be pointed out especially are: N-termini which are present in bacterial lipoprotein, such as, for example: Y-Ser-Ser-Ser-Asn, Y-Ile-Leu-Leu-Ala, Y-Ala-Asn-Asn-Gln, Y-Asn-Ser-Asn-Ser, Y-Gly-Ala-Met-Ser, Y-Gln-Ala-Asn-Tyr, Y-Gln-Val-Asn-Asn, Y-Asp-Asn-Ser-Ser, where Y can be one of the radicals listed under formula I to VII. Short forms (lipodipeptides, lipotripeptides or lipotetrapeptides) of these lipopentapeptides can also be used as membrane anchor compound. N-Palmitoyl-S-[2,3(bispalmitoyloxy)propyl]-cysteinyl-seryl-serine (Pam_3Cys-Ser-Ser), N-palmitoyl-S-[2,3(bispalmitoyloxy)propyl]-cysteinyl-seryl-glycine and N-palmitoyl-S-[2,3(bispalmitoyloxy)propyl]-cysteinyl-alanyl-D-isoglutamine are very particularly preferred.

Further compounds which are particularly preferred are compounds of the formulae I and III, in particular compounds of the formula I.

The substituent A is preferred to be sulfur or methylene,

sulfur is particularly preferred.

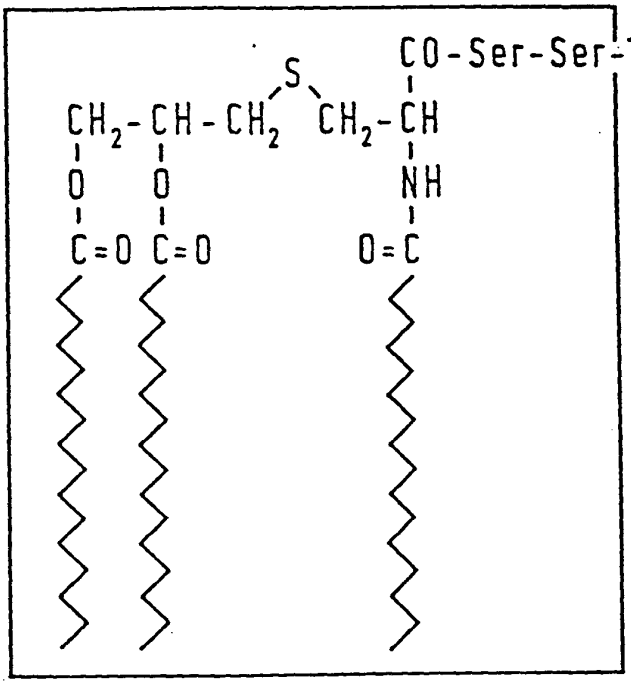
The substitu nts R, R' and R" are pr ferred to be alkyl radicals having 14 to 18 C atoms; alkyl radicals having 16 C atoms are particularly preferred.

The substituent X is preferred to be composed of 1 to 2 polar amino acid residues, the serine residue being particularly preferred.

Various proteins or partial protein sequences of pathogens which appear intracellularly or of viral, bacterial or parasite proteins or of tumor antigens which are recognized by killer T-cells, are suitable for the coupling to the membrane anchor compound for the vaccine according to the invention.

Such proteins or partial sequences (also referred to as killer T-cell epitopes) are distinguished by the fact that, together with MHC molecules (major histocompatibility complex), they are recognized by cytotoxic T-lymphocytes.

The vaccine according to the invention is suitable for the immunization against all pathogens which have killer T-cell epitopes, such as, for example, against adenoviruses, HIV, influenza viruses, LCMV, MCMV, hepatitis viruses, HTLV, FELV, Treponema pallidum, gonococcus, Bordetella pertussis, plasmodia, listeria, mycobacteria or leishmania. Killer T-cell epitopes which have been known previously are the partial sequences listed in the table below, the influenza nucleoprotein P_3CSS-NP 147-158 (R^-) and the HIV epitopes occupying a special position.



CO-Ser-Ser-T-Y-Q-R-T-R-A-L-V-T-G

Influenza nucleoprotein peptide NP 147-158 (R-)

Lipotripeptide Pam_3Cys-Ser-Ser

Organism	Protein	from - to	Restr.	sequence
Adenovirus Ad5E1A	-	-	Db	PSNTPPEI
HIV	env (gp 120)	381-392	HLA A2	(K)NCGGEFFYCNS
HIV	env (gp 120)	308-322	Dd	RIQRGPGRAFVTIGK
HIV	env (gp 120)	410-429	DR4	GSDTITLPCRIKQFINMWQE
HIV	gag (p17)	418-443	A2	KEGHQMKDCTERQANF
HIV	gag (p17)	446-460	A2	GNFLQSRPEPTAPPA
HIV	gag (p24)	193-203	A2	GHQAAMEMLKE
HIV	gag (p24)	219-233	A2	HAGPIAPGQMREPRG
HIV	gag (p24)	265-280	B27	KRWIILGLNKIVRMYC
Influenza	Nucleoprotein	82-94	HLA A2	MVVKLGEFYNQMM
Influenza	Matrix	57-68	HLA A2	KGILGFVFTLTV
Influenza	Nucleoprotein	335-349	B37 B44 A2 Aw69	SAAFEDLRVLSFIRG
Influenza	Hemagglutinin H3	58-73	H-2 Ad	ILDGIDCTLIDALLGD
Influenza	Hemagglutinin H3	58-73	H-2 Ad	ILDGIDCTLIDALLGD
Influenza	Hemagglutinin	181-204 103-123	H-2K;H-2K	-
Influenza	Nucleoprotein	365-379		SDYEGRLIQNSLTI
Influenza	Nucleoprotein	335-349	H-2b	IASNENMETMESSTL
Influenza	Nucleoprotein	384-393	HLA B27	RYWAIRTRSG
Influenza A/NT/60/68	Nucleoprotein	147-158	Kd	TYQRTRALV(R)TG
LCMV	Nucleoprotein	118-126	Ld Lq	RPQASGVYM
LCMV	-	278-286	H-2b	VENPGGYCL
-	-	277-293	H-2b	GVENPGGYCLTKWMILA
-	-	168-176	-	YPHFMPTNL
MCMV	-	161-179	Ld	GRLMYDMYPHFMPTNLGPS
P815	Tumor antigen P91A	12-24	Ld	ISTQNHRALDLVA
Plasmodium falciparum,	Circumsporo-zoite prot.	368-390	H-2K	KPKDELDYENDIEKKICKMEKCSC
berghei	"	249-260	Kd	NDDSYIPSAEKI
yoelii	-	276-288	Kd	NEDSYVPSAEQI
Hepatitis B	HBsAg	21-28	-	PLGFFPDH

With the aid of the vaccine according to the invention it moreover possible to mix various membrane anchor compounds coupled to various partial sequences in order to obtain a vaccine which is optimally adapted to a particular target. Furthermore, the corresponding mixture can additionally contain membrane anchor/active compound conjugates which stimulate the humoral immune response and additionally lead to the production of neutralizing antibodies (Vaccine 7, 29 - 33 (1989), Angew. Chem., Int. Ed. 24, 872 - 873 (1989)). Moreover it is also possible to couple various partial sequences covalently and combine them with a membrane anchor compound.

The invention furthermore relates to a process for the preparation of a synthetic vaccine which comprises bonding proteins or partial sequences of pathogens to the membrane anchor compound by a conjugating reaction. The conjugating reaction can, for example, be a condensation, addition, substitution, oxidation or disulfide formation. Conjugating methods which are preferred are shown in the examples. Further conjugating methods are described in the German Offenlegungsschrift 3,546,150 quoted above.

The preparation of membrane anchor compounds is likewise described in detail in the last-mentioned German Offenlegungsschrift.

The separation of diastereomers which may be necessary can be carried out by various methods as, for example, described in Hoppe-Seyler's Z. Physiolog. Chem. 364 (1983) 593.

The synthesis of the partial sequences to be employed in the membrane anchor/active compound conjugates can be carried out in various ways known from the literature, cf., for example, Wünsch et al. in Houben-Weyl, Vol. 15/1.2, Stuttgart, Thieme-Verlag or Wünsch in Angew. Chem. 83 (1971), E. Gross and J. Meienhofer (eds.), The Peptides, Vol. 1 (1979), 2 (1979), 3 (1981) and 5 (1983)

Academic Press, New York 7713 or the German Offenlegungsschrift 3,546,150. A preferred method for the preparation of a partial sequence and a conjugate is illustrated in more detail in Example 1.

Furthermore the invention relates to pharmaceutical preparations or preparations for veterinary medicine which contain conjugates of at least one membrane anchor compound and at least one partial sequence of one of the proteins or organisms mentioned. Normally no additional auxiliaries and excipients, or adjuvants are needed for the preparations according to the invention in addition to a solvent. However, it can be sensible in some cases to add auxiliaries and/or excipients of this type and, if desired, adjuvants to the preparations according to the invention (Anton Mayr, Gerhard Eißnen, Barbara Mayr-Bibrack, Handbuch der Schutzimpfungen in der Tiermedizin (Handbook of Vaccines in Veterinary Medicine), 1984, Verlag Paul Parey, Berlin-Hamburg).

The amount of vaccine which is necessary for a safe immunization of a warm-blooded animal depends on the species of warm-blooded animal, on the membrane anchor compound(s) and protein or the partial sequence(s) of the organism, immunity to which it is intended, and has to be determined empirically in each individual case.

The examples which follow are intended to illustrate the invention further.

Synthesis

Example 1

Synthesis of N-palmitoyl-S-[2,3(bispalmitoyloxy)-propyl]-cysteinyl-seryl-seryl-NP 147-158

The influenza A virus nucleoprotein peptide sequence was synthesized by solid phase peptide synthesis. Fmoc-amino

acids were used. The following side chain-protecting groups were used: Thr(tBu), Tyr(tBu), Arg(Pmc). 1 g of para-benzyloxybenzyl alcohol resin to which 0.5 mmol of Fmoc-Gly were bound, was used and the peptide sequence was synthesized by the following synthesis cycles. N-activation by 50% piperidine in DMF (1 × 10 min). Coupling of the subsequent amino acid for 30 min using BOP/HOBT [benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate/1-hydroxybenzotriazole] and diisopropylethylamine in DMF. Double couplings were carried out in each case using a 3-fold excess of Fmoc-amino acid and 4.5-fold excess of diisopropylethylamine (in each case in relation to free amino groups on the resin). After each double coupling the peptide resin was washed, in each case, three times with N-methylpyrrolidone, dichloromethane and N-methylpyrrolidone.

After the synthesis of the resin-bound influenza A virus nucleoprotein sequence, part of the peptide was isolated by cleavage with trifluoroacetic acid and tested for purity by means of HPLC, MS, amino acid analysis, analysis on chiral phase and sequence analysis. The HPLC analysis revealed a purity of more than 90%. After coupling two serine residues [Fmoc-Ser(tBu)] to the resin-bound peptide, the coupling of the tripalmitoyl-S-glycerolcysteine was carried out by the DIC/HOBT method. After four hours, one equivalent of N-methylmorpholine was added and, after one further hour, the lipopeptide-resin was washed. The lipopeptide was separated from 100 mg of resin by means of 2 ml of trifluoroacetic acid (containing 100 µl of thioanisole and 100 µg of thiocresol) in the course of one hour. In order to completely remove the Arg(Pmc) protecting groups, an additional subsequent treatment with trifluoroacetic acid was carried out at 50°C for 30 min. The filtrate was evaporated, the residue taken up in acetic acid and added to cold ether. The precipitated lipopeptide was washed 3 × with ether and freeze-dried from tert.-butanol/water in a ratio of 3:1.

Example 2

Synthesis of N-palmitoyl-S-[2,3-(bispalmitoyloxy)-propyl]-cysteinyl-seryl-seryl-NP (365-380)

The synthesis was carried out in analogy to Example 1. Fmoc-amino acids with the following side chain-protecting groups were used: Ser(tBu), Glu(OtBu), Thr(tBu). Asn was coupled without a side chain-protecting group by means of diisopropylcarbodiimide/HOBT. The initial resin used was Fmoc-Glu(OtBu)-p-benzyloxybenzyl alcohol-polystyrene, crosslinked with 1% divinylbenzene. The amount of Fmoc-Glu(OtBu) found was 0.45 mmol/g. The peptide and Pam_3Cys-Ser-Ser peptide was cleaved off from, in each case, 100 mg of resin using 2 ml of trifluoroacetic acid with the addition of 0.1 ml of thioanisole and 100 µg of thiocresol, in the course of 90 min. The sequence was confirmed by sequence analysis of the free peptide; a homogeneous peak containing more than 90% was determined by HPLC analysis. Amino acid analysis and testing for enantiomeric purity on chiral phase had the expected values as a result.

Activity tests

A)

3-month-old BALB/c inbred mice which had been bred under SPF conditions were immunized intravenously with 100 µg of Pam_3Cys-Ser-Ser-[NP 147-158]. (100 µg of Pam_3Cys-Ser-Ser-[NP 147-158], taken up in 300 µl of PBS, sonicated for 1 min). Afer 28 days, the mice were infected intranasally with 0.2 or 0.4 hemagglutinative units of influenza virus A/PR/8. In analogy, mice with 300 µl of PBS were administered intravenously were infected as a control. The course of the infection was monitored by means of daily controls of weight and by the survival rate. 11 of 12 control animals which had been infected with 0.4 hemagglutinative units died from the virus

infection after 11 days while only 4 of the 12 immunized animals died.

A further control group and a group immunized with Pam_3Cys-Ser-Ser-[NP 147-158] were infected with 0.2 hemagglutinative units of influenza virus. After 18 days, 40% of the control animals (4 of 10 animals) were still alive while 75% of the immunized animals were alive. On day 18 the weight difference between immunized animals and control animals was 4 g. The surviving animals of the control group continued to lose weight while the immunized animals slowly recovered from the infection.

B)
Cytotoxic T-cell activity of spleen cells from BALB/c mice after immunization with free peptide, virus or Pam_3Cys-Ser-Ser-peptide (Fig. 1)

BALB/c mice received by intravenous administration in 300 µl of PBS

a) 8×10^7 syngenic spleen cells preincubated with 1.6 µM of nucleoprotein peptide 147-158 (R-); (A, D, G),

b) 8×10^7 syngenic spleen cells preincubated with 160 µM of Pam_3Cys-Ser-Ser-[NP 147-158 (R-)] lipopeptide; (C, F),

c) 50 hemagglutinative units of influenza A virus PR/8/34; (B, E, H),

d) 100 µg of Pam_3Cys-Ser-Ser-[NP 147-158 (R-)]; (I).

After 6 days, the spleens were removed from the immunized or infected animals and these spleen cells were restimulated with peptide (A to F) or with syngenic stimulator cells infected with virus PR8 (G, H, I) for 5 days. For this purpose 2.5×10^7 cells in each case were cultivated in 10 ml of a-MEM medium (manufacturer: Gibco), supplemented with 10% fetal calf serum, 2-mercaptoethanol, glutamine and antibiotics with the addition of either 80 nM of NP 147-158(R-) peptide (A, F) or of 5×10^6 virus

PR8-infected, syngenic spleen cells which have been irradiated with 20 Gy (G, H, I). The infection of the stimulator and target cells was carried out as described (Eur. J. Immunol. 7, 630 - 635 (1977)).

The activity of the cytotoxic T-cells was determined by a ^{51}Cr-release standard test (Eur. J. Immunol. 137, 2.676 - 2.681 (1986)). Figures A, B, C and G, H, I show the CTL activity on untreated (Δ) or PR8-infected (Δ) P815 (MHC:H-2^d) target cells.

Figures D, E and F show CTL activity on P815 cells which were treated with various concentrations of free peptide at 37°C for 30 min. In this case a ratio of effector to target cell of 30:1 was used.

C)

Activity of cytotoxic T-cells after immunization of mice with Pam_3Cys-Ser-Ser-[NP 147-158] or Pam_3Cys-Ser-Ser-[NP 365-380] (Fig. 2)

BALB/c mice (Figures A, B) or (B6 × DBA/2) F1-mice (C, D) were immunized with influenza A virus (A, C) or with 100 μg of Pam_3Cys-Ser-Ser-[NP 147-158] (Figure B) or with 100 μg of Pam_3Cys-Ser-Ser-[NP 365-380] (Figure D). After 6 days, the spleens were removed and the spleen cells were, as described under B), stimulated in the presence of 0.8 μM of NP 147-158 peptide (A, B) or 0.8 μM of NP 365-380 peptide (C, D). The activity of the cytotoxic T-cells on untreated P815 target cells (Δ), on PR8-infected P815 target cells (Δ) and on P815 target cells preincubated with NP 147-158 peptide at 37°C for 90 min (■) was then assayed; likewise on untreated EL-4- (MHC H-2^d) cells (o), on PR8-infected EL-4 target cells (•) and on EL-4 cells preincubated with NP 365-380 peptide at 37°C for 90 min (♦).

D)

Test for MHC class I restriction and for specificity of

the immunization with lipopeptide (Fig. 3)

BALB/c mice (Figures A, B, C) or (B6 × DBA/2) F1-mice (Figures D-I) received i.v. in 300 µl of PBS
100 µg of Pam_3Cys-Ser-Ser-[NP 147-158(R-)] (Figures A, E, H) or
50 µg of Ser-Ser-[NP 147-158(R-)] (Figure B) or
50 µg of [NP 147-158(R-)] (Figure C) or
50 hemagglutinative units of influenza PR8 virus (Figures D, G) or 100 µg of Pam_3Cys-Ser-Ser-[NP 365-380] (Figures F, I).

Six days after the injection, the spleen cells were cultivated, as described in Example 2, with the addition of nucleoprotein 147-158(R-) peptide (Figures A - F), or nucleoprotein 365-380 peptide (Figures G - I). The activity of the resulting cytotoxic T-cells was determined against

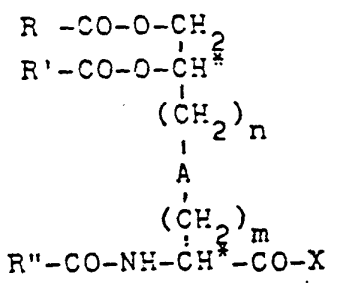
- untreated P815 target cells (Δ)
- P815 target cells preincubated with NP 147-158(R-) at 37°C for 90 min (■)
- P815 target cells preincubated with NP 365-380 at 37°C for 90 min (□)
- untreated EL-4 target cells (o)
- EL-4 target cells preincubated with NP 147-158(R-) at 37°C for 90 min (◊)
- EL-4 target cells preincubated with NP 365-380 at 37°C for 90 min (♦).

CLAIMS

1. A synthetic vaccine for the specific induction of cytotoxic T-lymphocytes which comprises a conjugate of at least one membrane anchor compound and a protein, containing at least one killer T-cell epitope, of a virus, a bacterium, a parasite or a tumor antigen, or at least one partial sequence containing at least one killer T-cell epitope of a viral, bacterial or parasite protein or of a tumor antigen.

2. The synthetic vaccine as claimed in claim 1 wherein the membrane anchor compound is a bacterial membrane lipoprotein.

3. The synthetic vaccine as claimed in claim 1 wherein the membrane anchor compound has one of the formulae below



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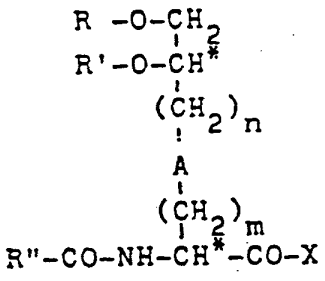

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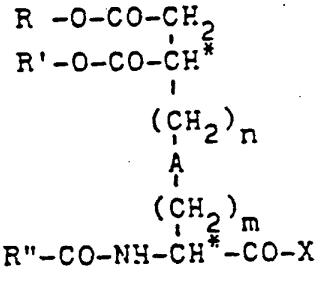

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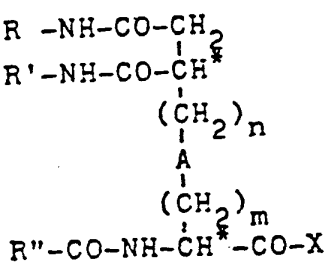

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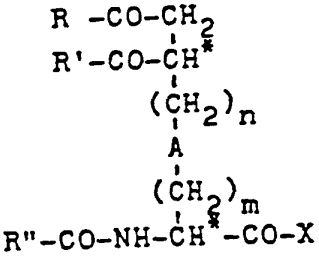

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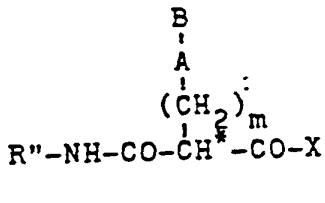

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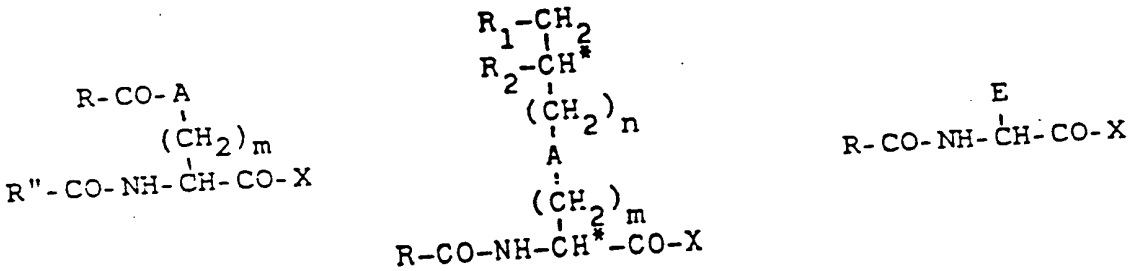

VII. VIII IX.

in which A may be sulfur, oxygen, disulfide (-S-S-), methylene ($-CH_2-$) or -NH-;
n = 0 to 5, m = 1 or 2;
C^* is an asymmetric carbon atom with an R- or S-configuration,
R, R′ and R" are identical or different and are hydrogen or an alkyl, alkenyl or alkynyl group having 7 to 25 carbon atoms, which can be substituted with hydroxyl, amino, oxo, acyl, alkyl or cycloalkyl groups, E in formula IX can be hydrogen or any desired side chain of a natural or artificial amino acid, B in formula VI can have the meaning of each of the $-(CH_2)_n-$(substituted alkyl) radicals listed in the formulae I - V, and R_1 and R_2 are identical or different and have the same meanings as R, R′ and R" but can also be -OR, -O-COR, -COOR, -NHCOR or -CONHR, where X is a chain of up to 10 amino acids to which the protein or the partial sequence of the viral, bacterial or parasite protein or of a tumor antigen is bonded, or is the protein or the partial sequence itself.

4. The synthetic vaccine as claimed in claim 1 wherein the membrane anchor compound is N-palmitoyl-S-2,3(bispalmitoyloxy)-propyl-cysteinyl-seryl-serine, the partial sequence being bonded to the terminal serine residue.

5. The synthetic vaccine as claimed in one or more of claims 1 - 4 wherein the protein or the partial sequence is derived from an adenovirus, HIV, influenza virus, LCMV,

MCMV, hepatitis virus, HTLV, FELV, Treponema pallidum, gonococcus, Bordetella pertussis or Plasmodium spec. or another pathogen containing a killer T-cell epitope.

6. The synthetic vaccine as claimed in one or more of claims 1 - 5 wherein a mixture of membrane anchor/active compound conjugates with various partial sequences is present.

7. The synthetic vaccine as claimed in claim 1 wherein, in addition to membrane anchor/active compound conjugates for the induction of cytotoxic T-lymphocytes, membrane anchor/active compound conjugates for the generation of neutralizing antibodies are also present.

8. A process for the preparation of a synthetic vaccine as claimed in one or more of claims 1 - 7 which comprises synthesizing a membrane anchor/active compound conjugate by known methods.

9. A pharmaceutical preparation or preparation for veterinary medicine for the induction of cytotoxic T-lymphocytes which contains a synthetic vaccine as claimed in one or more of claims 1 - 7, if desired in addition to customary auxiliaries or excipients and, if desired, in addition to further vaccines.

10. A synthetic vaccine as claimed in claim 1, substantially as hereinbefore described and exemplified.

11. A process as claimed in claim 8 for the preparation of a synthetic vaccine, substantially as hereinbefore described and exemplified.

12. A synthetic vaccine whenever prepared by a process claimed in claim 8 or 11.

13. A preparation as claimed in claim 9, substantially as hereinbefore described.

F.R. KELLY & CO.,
AGENTS FOR THE APPLICANTS.

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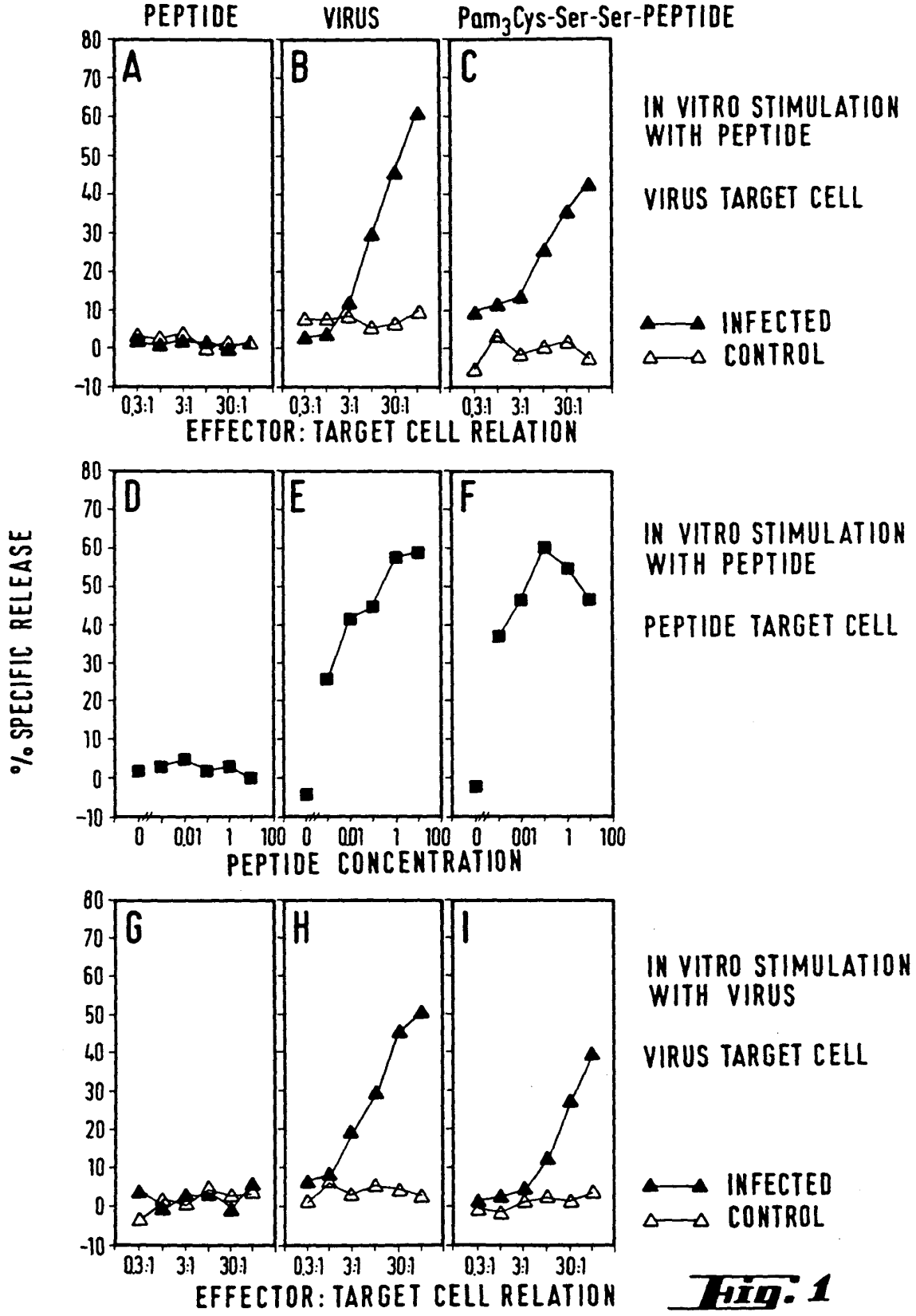

Fig. 1

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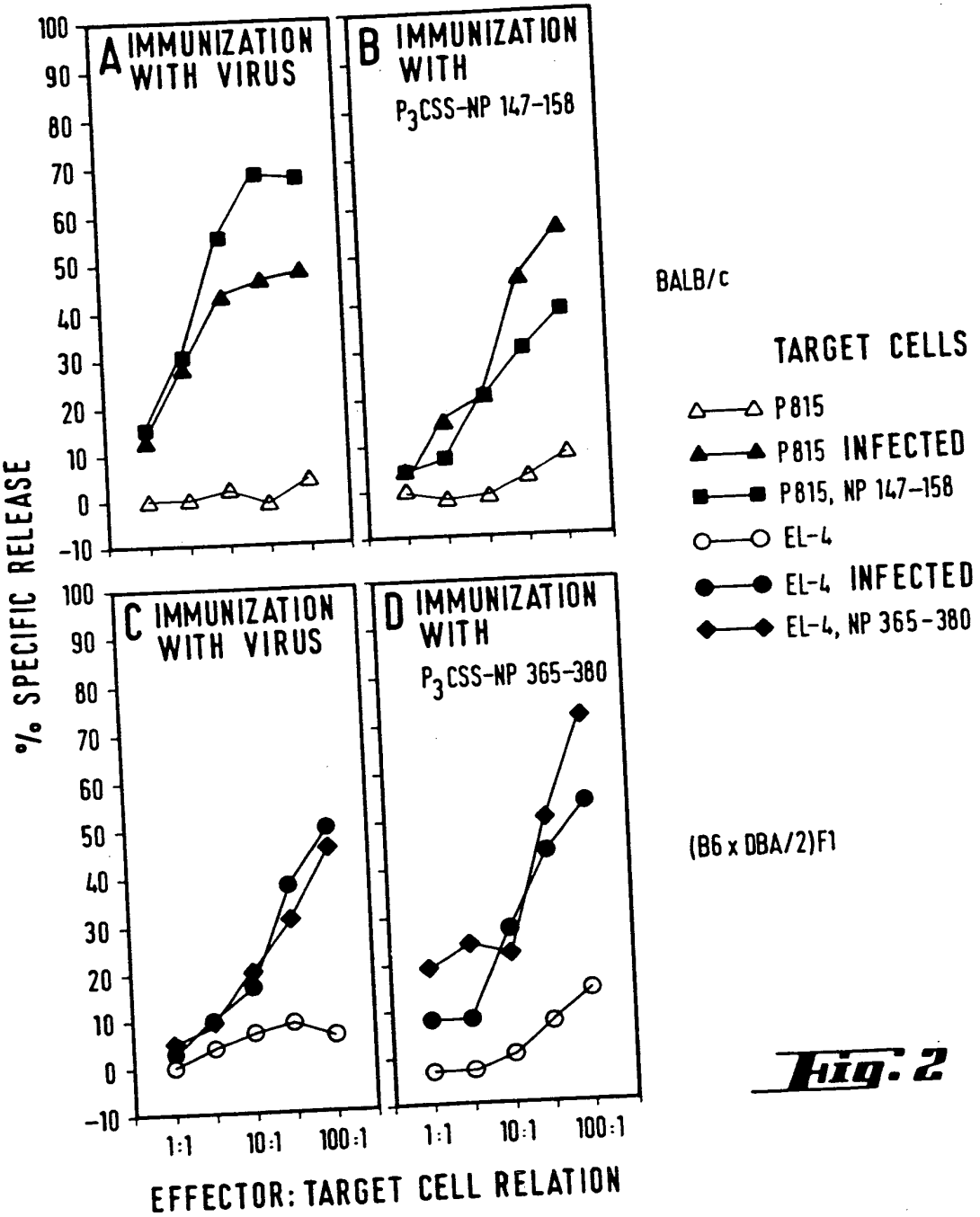

Fig. 2

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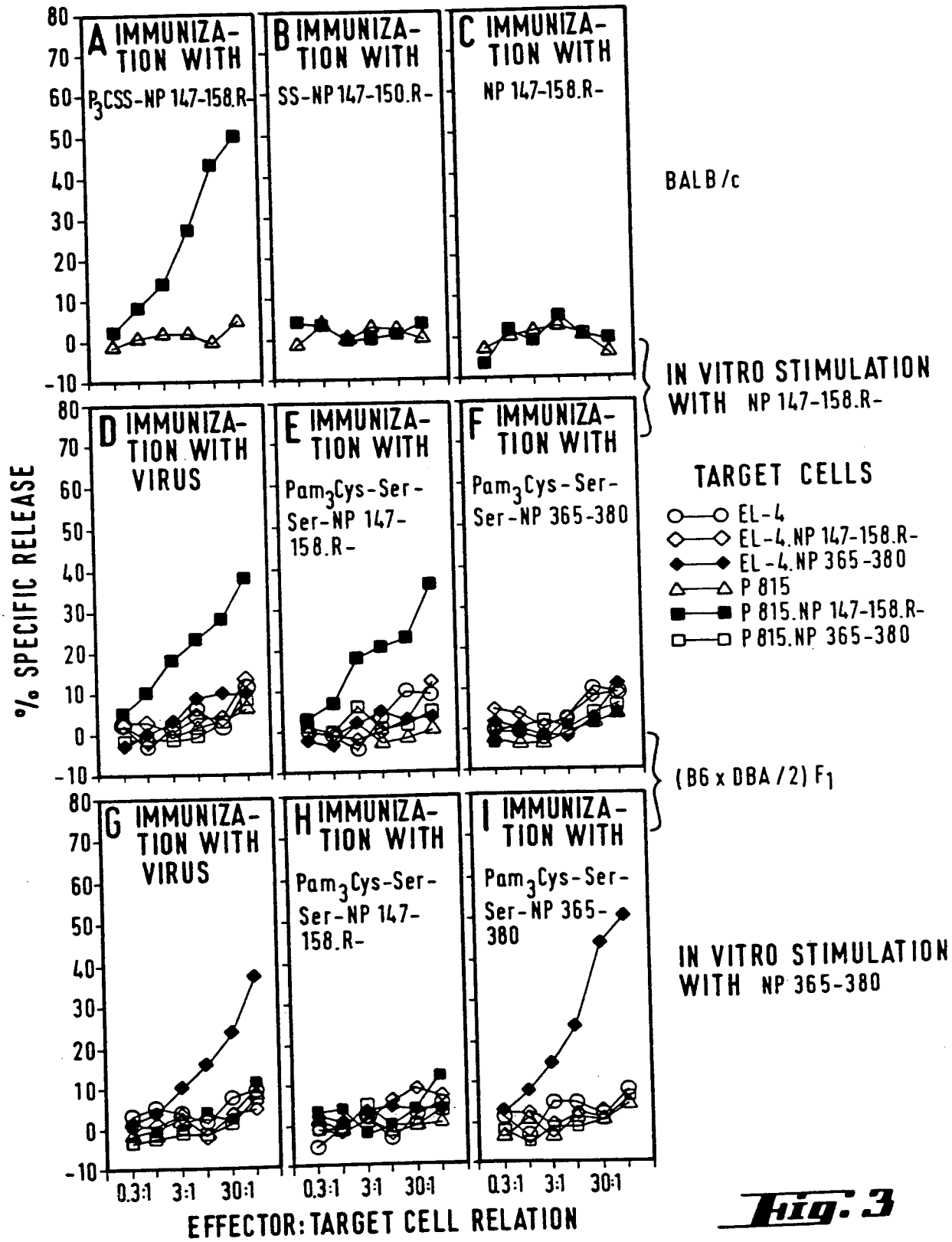

Fig. 3

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